

Metalloproteins and apolipoprotein C: candidate plasma biomarkers of T2DM screened by comparative proteomics and lipidomics in ZDF rats

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Research

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Abstract

Background: Early diagnosis of type 2 diabetes mellitus (T2DM) is still difficult. Screening of plasma biomarkers has great significance of optimizing diagnosis and predicting the complications of T2DM.

Methods: We used a special diet, Purina #5008, to induce diabetes in Zucker leptin receptor gene-deficient rats (fa/fa) to establish Zucker diabetic fatty (ZDF) rats, simulating the early stage of T2DM. The differentially expressed proteins (DEP) and lipids (DEL), as potential biomarkers, were screened to compare the plasma expression levels in ZDF rats and their basic diet-fed wild-type controls (fa/+) by Tandem Mass Tags (TMT) and liquid chromatography-tandem mass spectrometry.

Results: These two groups had different plasma proteins and lipids profiles consisting of 84 DEPs and, 179 DELs identified in the positive ion mode and 178 DELs in the negative ion mode, respectively. Enrichment analysis of these different indicators showed that oxidative stress, insulin resistance and metabolic disorders of glycan and lipid played an important role in generating the difference. Some markers can be used as candidate biomarkers in prediction and treatments of T2DM, such as ceruloplasmin, apolipoprotein C-I, apolipoprotein C-II, apolipoprotein C-IV.

Conclusion: These plasma differences help to optimize the diagnosis and predict the complications of T2DM, although this remains to be verified in the crowd. Trace elements related-metalloproteins, such as ceruloplasmin, and lipid metabolism and transport-related apolipoprotein C are expected to be candidate biomarkers of T2DM and should be given more attention.

1. Background

Diabetes, one of the leading causes of death in the world, is a chronic, metabolic disease characterized by elevated levels of blood glucose. Based on the investigation from the International Diabetes Federation (IDF) Atlas, about 425 million adults worldwide aged 20–79 years are affected by diabetes in 2017 [1]. Most of the diabetics (90%-95%) suffering from type 2 diabetes mellitus (T2DM) could benefit directly from early diagnosis and treatments [2]. However, unfortunately, half of the patients may be undiagnosed due to the lack of early detection [1]. The conventional methods based on blood glucose testing need to be improved or supplemented with other diagnostic methods. Moreover, with the obese population and the prevalence of T2DM growing rapidly [3-6], the necessity for prompt diagnosis or prediction of T2DM becomes more urgent.

The development of diseases is accompanied by metabolic changes, and existing studies have shown that biomarkers in plasma and urine can predict the occurrence of some chronic diseases [7-9]. This helps to optimize the diagnostic method and to predict related complications. The biomarkers research regarding diabetes nephropathy, a serious complication of diabetes, has made great progress [10, 11], however, study of early diagnosis of T2DM is still limited [12]. To a certain extent, this is due to that the

presence of severe metabolic disorders and signs of microvascular damage in the stage of diabetic complications help in the selection of markers; while slight changes in blood glucose and other metabolites in the early stages of diabetes are not likely to be discovered by epidemiological studies. Therefore, screen potential biomarkers in diabetes animal models is an indispensable step for improving early diagnosis of T2DM.

ZDF rats are commonly used as spontaneous T2DM animal models and are highly recognized in the development of diabetes drugs [13-16]. Due to the defection of leptin receptor-gene, they show characteristics such as obesity, hyperglycemia, insulin disorders and dyslipidemia in the case of special diet induction, which closely match the pathological characteristics of T2DM patients. This diet-only modeling method is similar to natural development of T2DM in human and does not change the physiological state of rats which may change in experimental diabetes animal models due to drug or surgery. This is of great significance to the screening of candidate biomarkers of T2DM and provides feasibility for our study. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology also provides a reliable mean for plasma proteomics and lipidomics. In preliminaries screening of plasma differentially expressed proteins (DEP) and lipids (DEL) in Zucker diabetic fatty (ZDF) rats, this study provides an important reference for screening and verification of T2DM plasma biomarkers in the crowd.

2. Methods

2.1. Animals and groups

Zucker leptin receptor gene-deficient rats (fa/fa) and their littermate wild-type rats (fa/+) (male, 8 weeks of age, SPF VAF/Elite) were supplied by Charles River in Beijing, China. All animals were kept in a barrier system. The animal room was maintained at approximately 22°C and 50% humidity with a 12 hours light/dark cycle. Food and drinking water were available. Purina #5008 (protein 23.5%, fat (ether extract) 6.5%, fat (acid hydrolysis) 7.5%, fiber (crude) 3.8%, nitrogen-free extract (by difference) 49.4%, ash 6.8%; gross energy 4.15 kcal/gm. Calories provided by the calorogenic nutrients: protein 26.849%, fat (ether extract) 16.710%, carbohydrates 56.441%.) was utilized to induce obesity and diabetes in Zucker leptin receptor gene-deficient rats (fa/fa). Simply, they were fed by Purina #5008, starting at 8 weeks of age, for 3 weeks. Blood glucose >11.1 mmol/L was used as the standard of successfully modeling of ZDF rats [17]. In order to avoid the die's effects on plasma, the ZDF rats were maintained on a basic diet (crude protein ≥18%, crude fat ≥4%; gross energy 3.40 kcal/gm. Calories provided by the calorogenic nutrients: protein 23.07%, fat (ether extract) 11.85%, carbohydrates 65.08%.) for one week, that was the 12th weeks. The wild-type rats (fa/+) were kept on a basic diet all along. By the end of the 12th week, all animals were fasted for 12 hours, anesthetized and bled from the abdominal aorta using EDTAK₂ anticoagulation tubes. Plasma was collected after standing and centrifugation, and then stored at -80°C until detection. Three samples from each of the ZDF group and their basic diet-fed littermate wild-type group were labeled with TMT to analyze the proteins in plasma by LC-MS/MS. And six from each were used to analyze the liquids by LC-MS/MS. All animals were treated according to the NIH Guide for Care and Use

of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of Shandong University.

2.2. Proteomic TMT labeling and LC-MS/MS analysis

Proteomic TMT labeling technology used isotopically labeled peptides to analyze the protein levels in groups by high-precision mass spectrometer [18]. The experimental procedures in our study included: extraction, quantification, detection, removal of peak protein, enzyme digestion and desalting [19], labeling, fraction separation and mass spectrometry [20], etc. Reagents and procedures were described in the Additional file 1.

2.3. Proteins identification and screening of differentially expressed proteins (DEP)

The mass data was directly imported into Proteome Discoverer 2.2 for database search. The database we used was the Uniprot (Accessed 18 January 2019, *Rattus Norvegicus*, 36090 sequences). Analysis parameters were described carefully in the Additional file 1. Peptides with a confidence of more than 95% were peptides spectrum matches (PSMs). Proteins containing at least one unique peptide were trusted proteins. We screened the results and retained only the PSMs and trusted proteins. FDR validation was also performed to remove peptides and proteins with P -value above 5%. Relative protein quantification was performed based on the peak area. The ratio of the mean quantization of the ZDF group to their basic diet-fed littermate wild-type group was the fold change (FC). We considered $FC > 1.2$ and $P < 0.05$ as DEPs.

2.4. DEPs enrichment analysis

Gene Ontology (GO), Cluster of Orthologous Groups of proteins (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations, and Inter Pro (IPR) annotations [21-25] were performed to fully understand the functional properties of DEPs.

GO function enrichment analysis was carried out to identify the functional process of the DEPs in biological processes (BP), cell composition (CC) and molecular function (MF) by hypergeometric verification. KEGG pathway enrichment analysis was also conducted for exploring the causes of DEPs and the mechanisms of T2DM. $P < 0.05$ was identified as the significant difference.

2.5. Protein-protein interaction (PPI) network analysis of DEPs

PPI network analysis of the DEPs was constructed from the STRING (<https://string-db.org>) and visualized by Cytoscape (version 3.7.1). The Molecular Complex Detection (MCODE, version 1.31) app in Cytoscape was used to analyze the modules in the network.

2.6. Lipidomic LC-MS/MS analysis

The LC-MS/MS technique was used for lipidomics research. The experimental procedures included: lipid extraction, LC-MS/MS detection [26-30], etc. The reagents and procedures were detailed in the Additional

file 1.

2.7. Lipids identification and screening of differentially expressed lipids (DEL)

Progenesis QI (Waters) was used to identify lipids and multivariate statistical analysis. Lipidmaps (<http://www.lipidmaps.org>), HMDB (<http://www.hmdb.ca>), NIST (<https://chemdata.nist.gov>) and an in-house lipid database of Novogene Bioinformatics Technology Co. Ltd were used for identification. Reagents and procedures are also described in the Additional file 1. The multivariate statistical analyses used to reveal the differences included principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The variable importance in the projection (VIP) of the first principal component of the PLS-DA model was combined with P of T-test to screen DELs. We considered $VIP > 1.0$, $FC > 2.0$ and $P < 0.05$ as DELs.

2.8. Correlation analysis of proteomics and lipidomics

According to the order of FC, we selected the top 50 DEPs and the top 20 DELs for statistical correlation analysis of expression levels to explore the consistency of the proteomic and lipidomic data. We also conducted KEGG pathway enrichment analysis on DELs, reviewed and compared the results of DEPs and DELs. The pathways in which both proteins and lipids were enriched had received particular attention.

3. Results

3.1. Purina #5008 diet-induced irreversible diabetes in Zucker leptin receptor gene-deficient rats

After fed by Purina #5008 for 3 weeks, up to 11 weeks old, Zucker leptin receptor gene-deficient rats (fa/fa) developed obesity and elevated blood glucose (Fig. 1). And this early diabetic state was not corrected by one week's basic diet, that was when they were 12 weeks old ($n=10$, paired T-test in 11 W and 12 W, $P=0.259$).

3.2. Screening of DEPs and their enrichment analysis

We identified a total of 697 proteins (Fig. 2a). Quantitative data and annotation results of these proteins were detailed in the Additional file 2. Among all the identified proteins, 25 were significantly up-regulated ($FC > 1.2$ and $P < 0.05$) and 59 were markedly down-regulated ($FC < 0.83$ and $P < 0.05$) (Fig. 2b). The criteria used in our study was appropriate, which was confirmed by the hierarchical clustering of DEPs (Fig. 2c).

GO function enrichment analysis gave significant enriched GO function entries in the DEPs compared to all identified proteins (Fig. 2d), defining the biological function of the DEPs. GO biological process (BP) analysis found that the DEPs were mainly enriched in multicellular organism development, system development, regulation of bone mineralization, cell adhesion, homophilic cell adhesion via plasma membrane adhesion molecules, negative regulation of cellular process, regulation of biological process, and oxidation-reduction process. In the cell composition (CC) part, the DEPs were involved in the

extracellular matrix and extracellular region. In the molecular function (MF) section, the DEPs joined in the calcium ion binding, metal ion binding, lyase activity, hydro-lyase activity, magnesium ion binding, enzyme activator activity.

KEGG pathway enrichment analysis demonstrated that the DEPs were enriched in proteoglycans in cancer, ECM-receptor interaction, HIF-1 signaling pathway, endocrine resistance, RNA degradation, which indicated that those above and T2DM share the same molecular pathways.

3.3. PPI analysis of DEPs raised the need for lipidome

There were 69 common proteins and 89 interactions when we matched the 84 DEPs with proteins in the STRING database (*Rattus Norvegicus*). The results were described in detail in the permalink: STRING (<https://version-11-0.string-db.org/cgi/network.pl?networkId=fOIDKdXKqgFI>. Accessed 28 May 2019). A network containing 15 up-regulated proteins and 33 down-regulated proteins was performed after removing unconnected nodes (Fig. 2e). Four significant modules were constructed by MCODE, one of which was associated with lipid metabolism and transport. Preliminary analysis of these proteins in this module suggested there were some changes in the plasma lipids. So, we conducted plasma lipidomics.

3.4. The screening of DELs

We identified 1000 lipids in the positive ion mode, of which 153 were substantially up-regulated (VIP >1.0, FC >2.0 and $P < 0.05$) and 26 were significantly down-regulated (VIP >1.0, FC <1.5 and $P < 0.05$). In the negative ion mode, we identified 1291 lipids, of which 139 were substantially up-regulated and 39 were significantly down-regulated. The quantitative data and statistical analysis results of these lipids were detailed in the Additional file 3. We obtained lipid classification by matching the screened DELs with the Lipidmaps database (<http://www.lipidmaps.org>), removed unmatched entries and counted the number of DELs accompanied by each classification. The top categories are Glycerolipids (GL), Glycerophospholipids (GP), Fatty Acyls (FA), and Sphingolipids (SP) in the positive ion mode. And in the negative ion mode, they are GP, FA, SP, and GL (Fig. 3a). The plasma lipid profile of ZDF rats was different from their basic diet-fed littermate wild-type control (Fig. 3b), and like the plasma protein profile, it could distinguish the state of T2DM.

3.5. Correlation analysis suggested the main reason for the differences

The expressions of the top 50 DEPs and the top 20 DELs are strongly correlated. A simple statistical display of the absolute value of the Pearson correlation coefficient is as follows: Mean \pm SD and Median [IQR], 0.90 ± 0.05 and $0.91 [0.07]$ in the positive ion mode and 0.86 ± 0.09 and $0.88 [0.11]$ in the negative ion mode. Correlation analysis showed a high consistency between DEPs and DELs (Fig. 3c). Please refer to the Additional file 4 for the correlation analysis heatmap with detailed DEPs/DELs annotations.

Based on this, we conducted KEGG pathway enrichment analysis on DELs as did on DEPs aiming to find the main reasons for the differences. The analysis prompted that DELs were enriched in purine metabolism, biosynthesis of alkaloids derived from histidine and purine in the positive ion mode, and in

synthesis and degradation of ketone bodies in the negative ion mode. The original *P*-value was then corrected by hypergeometric verification, and the KEGG pathway enrichment results of both DEPs and DELs were compared and reviewed (Fig. 4). We found that metabolism disorder of glycan and lipid plays a significant role in the pathogenesis of T2DM. Besides, the enrichment results of DEPs also suggested oxidative stress and insulin resistance were related to the changes. Table 1 displays the candidate biomarkers related to the mechanism of these differences.

Table 1 Candidate biomarkers of T2DM suggested by KEGG pathway enrichment analysis

Pathogenesis (suggested by KEGG pathways)	Candidate biomarkers of T2DM	
	Up-regulated proteins	Down-regulated proteins
Oxidative Stress		ceruloplasmin
		extracellular superoxide dismutase [Cu-Zn]
		glutathione peroxidase 6
Insulin Resistance	glycogen phosphorylase, liver form	insulin-like growth factor 1, isoform CRA_b
	60 kDa heat shock protein, mitochondrial	
Glycan Biosynthesis and Metabolism	glyceraldehyde-3-phosphate dehydrogenase	
	4-trimethylaminobutyraldehyde dehydrogenase	
Lipid Metabolism	apolipoprotein C-I	apolipoprotein M
	apolipoprotein C-II (Predicted)	very low-density lipoprotein receptor
	apolipoprotein C-IV	

4. Discussion

Our study here showed that ZDF rats (fa/fa) and their basic diet-fed littermate wild-type rats (fa/+) exhibited different plasma proteins and lipids profiles which could distinguish the diabetic status of rats clearly by the hierarchical clustering of DEPs/DELs. GO function enrichment analysis demonstrated that DEPs were in the extracellular, which gave these proteins the potential to become plasma biomarkers. Furtherly, KEGG pathway enrichment analysis of DEPs and DELs revealed the related mechanisms of T2DM, such as oxidative stress, insulin resistance and metabolic disorders. This was consistent with previous researches [31-33]. Some differentially expressed indicators and their role in KEEG pathways led us to believe that they had the potential to be biomarkers, as follows: Down-regulated ceruloplasmin,

extracellular superoxide dismutase [Cu-Zn] and glutathione peroxidase 6 indicated a decrease in antioxidant level [34, 35]. Up-regulated glycogen phosphorylase (liver form), 60 kDa heat shock protein (mitochondrial), and down-regulated insulin-like growth factor 1 (isoform CRA_b) proved a significant insulin resistance [36-38]. Up-regulated glyceraldehyde-3-phosphate dehydrogenase and 4-trimethylaminobutyraldehyde dehydrogenase showed an increasing degree of plasma glycolysis [39]. Up-regulated apolipoprotein C-I and apolipoprotein C-II illustrated blood low-density lipoproteins accumulated in the blood, thereby increasing the risk of cardiovascular complications in diabetes [40, 41].

Importantly, we found two interesting points in these screened biomarkers.

Firstly, three oxidative stress-related markers that we screened, ceruloplasmin, extracellular superoxide dismutase [Cu-Zn] and glutathione peroxidase 6, are all trace elements related-metalloproteins. Ceruloplasmin stores approximately 95% of copper in the blood in a non-diffused state [42] and is linked to iron metabolism [43]. More than half of the patients with aceruloplasminemia (ACP), an autosomal recessive genetic disease caused by mutations in the gene encoding ceruloplasmin, have diabetes as their earliest symptom [44]. And some epidemiological studies use ceruloplasmin to indicate diabetes nephropathy progresses [45-49]. Each subunit of extracellular superoxide dismutase [Cu-Zn] contains a copper ion and a zinc ion, and each of the four subunits of glutathione peroxidase 6 contains a single selenium ion. These metal trace elements play a major part in maintaining the normal function of these proteins [50-52]. So, our study provides evidence for the association of T2DM with trace elements, such as copper, zinc, iron, selenium, through metalloproteins.

The second point is a question of lipid metabolism and transport. A significant module of the DEPs PPI network, which contains three up-regulated proteins, apolipoprotein C-I, apolipoprotein C-II (Predicted) and apolipoprotein C-IV, and two down-regulated proteins, apolipoprotein M and very-low-density lipoprotein receptor, suggests metabolism and transport disorder of lipid. Hierarchical clustering of DELs proves this. Since plasma lipids are greatly influenced by diet, we use the basic diet to feed ZDF rats for one week and all animals are fasted for 12 hours before collecting plasma samples. And because of this, we don't screen biomarkers in DELs. It is noteworthy that our results show the association between the apolipoprotein C and T2DM. Since there are limited studies in this area [53, 54], we will pay more attention to the changes in apolipoprotein C during the progress of T2DM in the future.

The pathogenesis of T2DM is complicated. Multi-omics study helps to profoundly understand the molecular mechanisms and explores the possible directions in diagnosis and treatment of it. Screening of plasma biomarkers has unparalleled advantages, as the plasma is more stable and more readily available compared to urine and tissues, respectively [55, 56]. We screened the potential biomarkers of T2DM by comparing the plasma proteins and lipids expression levels in ZDF rats (fa/fa) and their basic diet-fed littermate wild-type controls (fa/+). The comparison method we adopted fully considered the influence of genetics and environments. Although this comparison will overestimate the role of the genetic effects of the leptin receptor gene in T2DM and increase the difficulty of comparison with other similar studies [57, 58], we believe this is a simple and effective comparison strategy when the population's genetic background is not known clearly. So far, very limited studies have been performed

with regard to detection of plasma proteins and lipids profiles in ZDF rats. Therefore, this study may provide a novel strategy to characterize the molecular mechanism of T2DM and search for potential biomarkers [54, 59, 60], despite the fact that this is only at the animal level. It is notable that the samples number is small, although this is sufficient for LC-MS/MS analysis. Increasing samples and verifying the predictability of these candidate biomarkers are the focus of our next work.

5. Conclusions

Differentially expressed proteins and lipids in plasma are helpful for early diagnosis and predict the complications of T2DM. Trace elements related-metalloproteins, such as ceruloplasmin, and lipid metabolism and transport-related apolipoprotein C are important in the progression of diabetes and are expected to be candidate plasma biomarkers of T2DM.

Abbreviations

T2DM	type 2 diabetes mellitus
ZDF	Zucker diabetic fatty rats
DEP	differentially expressed proteins
DEL	differentially expressed lipids
TMT	Tandem Mass Tags test
LC-MS/MS	liquid chromatography-tandem mass spectrometry
PSMs	peptide spectrum matches
FC	fold change
PPI	protein–protein interaction
PCA	principal component analysis
PLS-DA	partial least squares discriminant analysis
VIP	variable importance in the projection
ACP	aceruloplasminemia
QC	quality control

Declarations

Ethics approval and consent to participate

All animals were treated according to the NIH Guide for Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of Shandong University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WS was responsible for analysis, visualization, writing of the manuscript; LZY made substantial contributions to data acquisition; WYX and ZTR participated in the data interpretation and made substantial revisions in the first draft; HXD contributed to the reception and design of the work. All authors read and approved the final manuscript.

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Figures

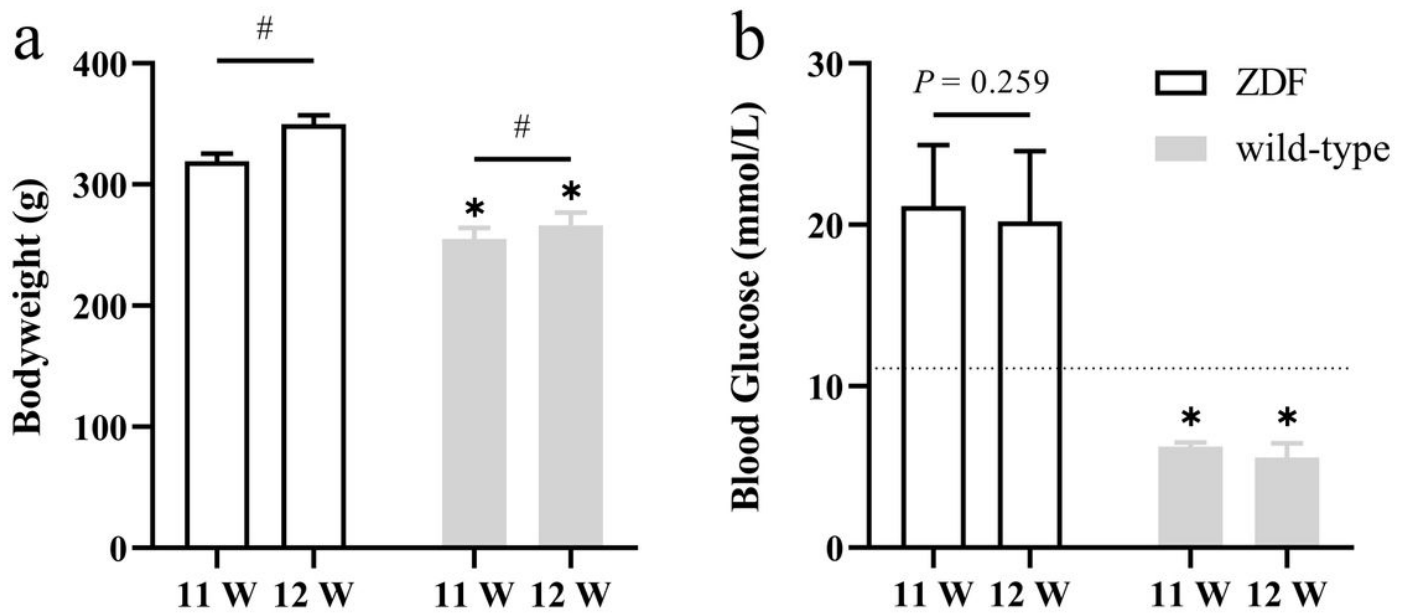


Figure 1

Purina #5C08 diet-induced irreversible diabetes in Zucker leptin receptor gene-deficient rats. a Bodyweight of the rats. b Blood glucose of the rats. The dotted line in the figure represents the standard for successful modeling of ZDF, the blood glucose >11.1 mmol/L. (n = 10. #: $P < 0.05$, paired T-test in 11 W and 12 W. *: $P < 0.05$, paired T-test in ZDF and their basic diet-fed littermate wild-type control at the same time point.)

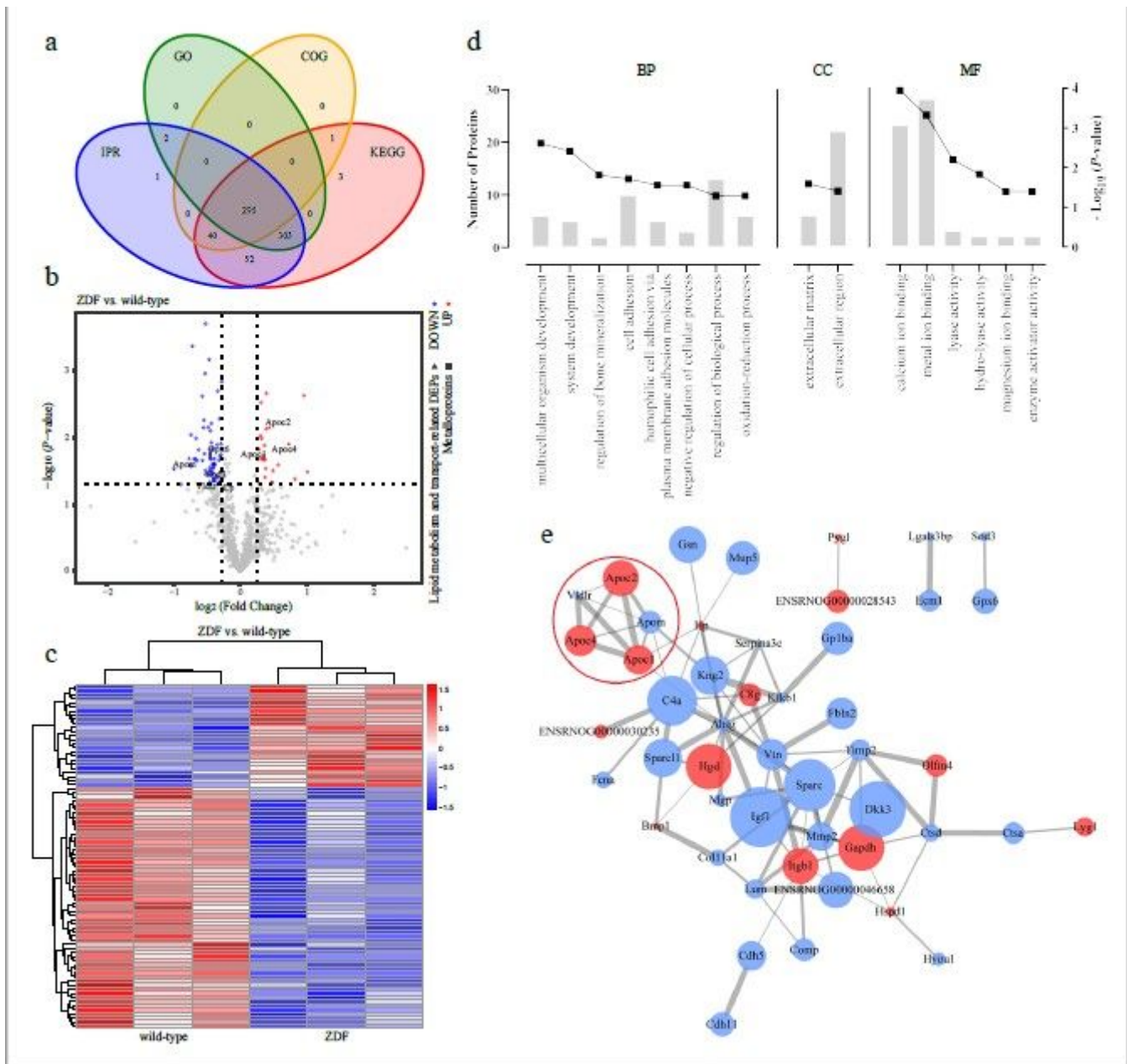


Figure 2

Visualization of differentially expressed proteins (DEP) and enrichment analysis. a Function annotations Venn graph of all the identified 697 proteins. b Volcano plot of DEPs. Gray in the Volcano plot indicate the proteins with insignificant differences, red indicates up-regulated and blue indicates down-regulated. We use triangles and squares to highlight the lipid metabolism and transport-related DEPs and metalloproteins, respectively. c Heatmap of DEPs. Each row is corrected for the Z value. Longitudinal is the clustering of samples and horizontal is the clustering of proteins. The heatmap with annotations are provided in the Additional file 4. d Histogram of GO enrichment analyses results. The entries in the histogram are arranged from left to right according to the degree of enrichment, and the curves show the change of enrichment degree. e Protein-protein interaction network of DEPs. Each node represents a protein. The up-regulated protein is in red and down-regulated protein is blue. The size of each node is proportional to the $-\log_{10} P$ -value. The edges represent protein-protein interactions. The width of the edge

is proportional to the combined-score in STRING. The module circled by the red line is associated with lipid metabolism and transport.

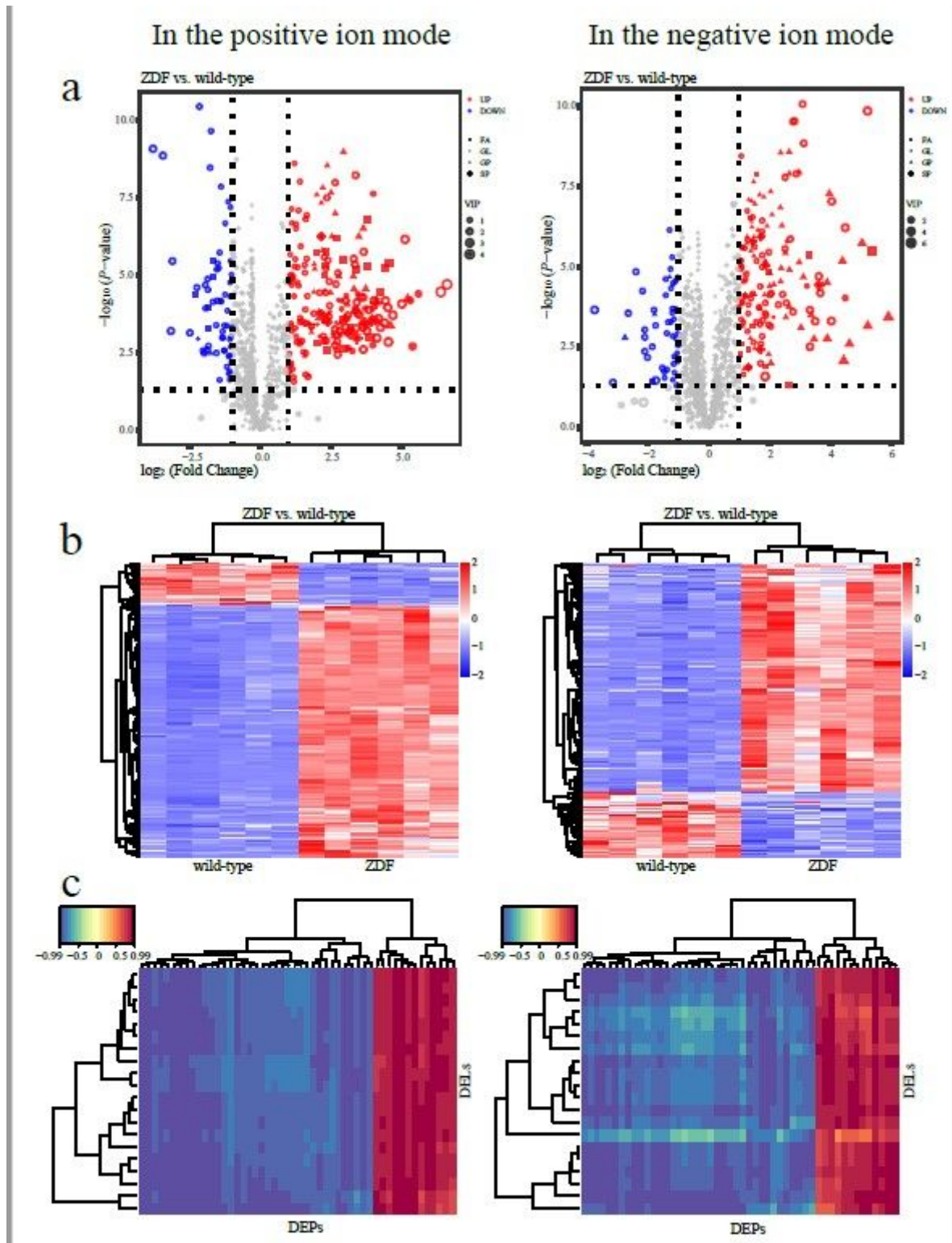


Figure 3

Visualization of differentially expressed lipids (DEL) and their correlation with DEPs. a Volcano plot of DELs. Gray in the Volcano plot represents a lipid with no differential expressions, red represents up-regulated and the blue represents down-regulated. We use different shapes to highlight the lipid

categories that changed significantly. The size represents the variable importance in the projection (VIP).
 b Heatmap of DELs. Each row is corrected for the Z value. The heatmap with annotations are provided in the Additional file 4. c Correlation analysis heatmap of the top 50 DEPs and the top 20 DELs according to the order of FC. The redder the color, the stronger the positive correlation; the bluer the color, the stronger the negative correlation. The heatmap with annotations are provided in the Additional file 4. Fig. 4 Visualization of KEGG pathway enrichment analysis of DEPs and DELs. a Bubble chart of KEGG pathway enrichment analysis of DEPs. Abscissa represents the enrichment degree, the ratio of differences to the number of backgrounds identified in the pathway. The color is proportional to the $-\log_{10}$ P-value and the size represents the number. b Result of DELs in the positive ion mode. c Result of DELs in the negative ion mode. (DEPs results show only a part, DELs results are comprehensive.)

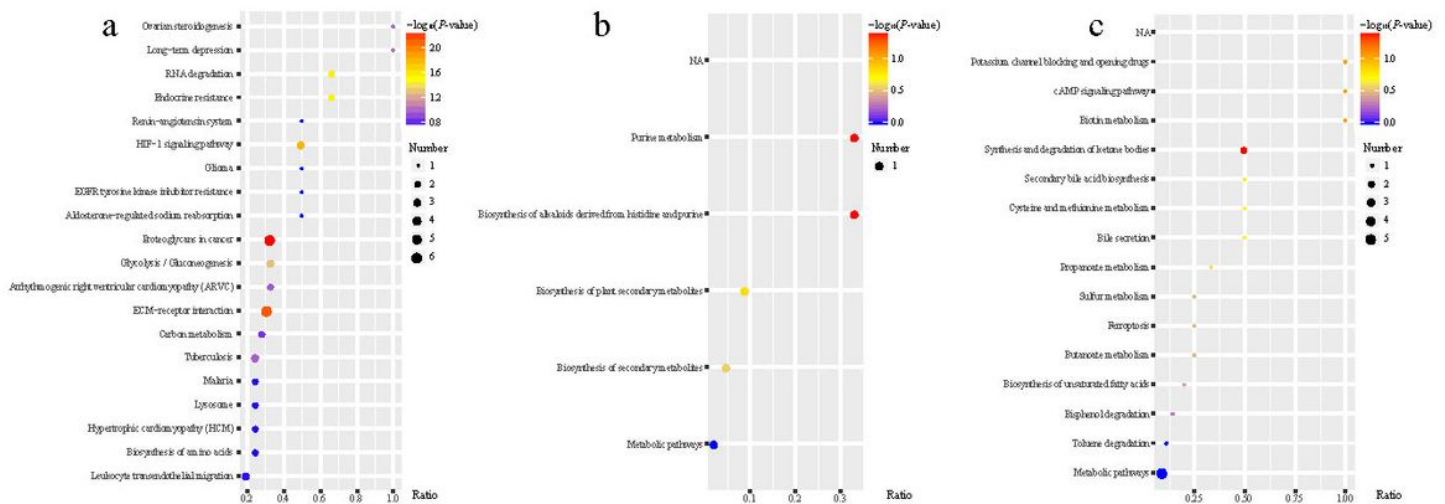


Figure 4

Visualization of KEGG pathway enrichment analysis of DEPs and DELs. a Bubble chart of KEGG pathway enrichment analysis of DEPs. Abscissa represents the enrichment degree, the ratio of differences to the number of backgrounds identified in the pathway. The color is proportional to the $-\log_{10}$ P-value and the size represents the number. b Result of DELs in the positive ion mode. c Result of DELs in the negative ion mode. (DEPs results show only a part, DELs results are comprehensive.)

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